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Current Topics

Tyrosinase and Glycoprotein Folding: Roles of Chaperones That Recognize Glycans†

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Glycoprotein folding is regulated in the ER by multiple mechanisms destined to enable the polypeptide chain to acquire the unique 3D structure of the native glycoprotein. It is widely held that the efficiency of the folding depends on the interaction of the folding polypeptide with ER-resident molecular chaperones. There certainly are distinct differences among the ER chaperones that can be described in terms of specificity toward the recognition elements exposed by the nascent polypeptide chain. Whereas most of the chaperones identify regions in the polypeptide moiety, two chaperones, calnexin and calreticulin, are known to modulate the folding of the nascent chain through their interaction with the attached *N*-glycans. Calnexin/calreticulin interaction with the nascent glycoprotein is mediated by the monoglucosylated *N*-glycans carried by every single glycoprotein in the early stages of the *N*-glycan processing. The important finding that a lectin-like interaction promotes efficient folding provides glycobiology with a fundamental concept for understanding the roles of N-glycosylation in glycoprotein function. Most strikingly, the early *N*-glycan processing stages are related to the chaperone-mediated folding of the nascent polypeptide, thus contributing to the quality control mechanism in the ER.

A number of studies have used in vitro translation systems to highlight specific interactions between the chaperones calnexin and calreticulin and the newly synthesized glycoproteins (*1*, *2*). These glycoproteins depend on their interaction with the ER lectins in terms of competence for export from the ER and resistance to degradation (*3*, *4*). Therefore, export from the ER is taken as an indication of secretory proteins having reached their native state (*5*).

How does this concept stand up when we look for confirmation in in vivo systems? Recent in vivo studies using mouse melanoma tyrosinase show that although tyrosinase activity and folding are absolutely dependent on the interaction with calnexin, the bypass of this quality control does not impair the intracellular transport of the glycoprotein (*6*, *7*). Two important points follow from this. First, there is the confirmation that calnexin/calreticulin association with the nascent glycoprotein chain is crucial for the correct folding in vivo, as has been shown for in vitro systems. Second, it appears that the export competence concept might not apply to all proteins and in particular to metalloglycoproteins such as tyrosinase.

Tyrosinase is a good model to study the relation between calnexin interaction and glycoprotein function (*7*, *8*). The enzymatic activity of tyrosinase (which can be easily measured on a gel) can be used as a measure of protein conformation. This enzymatic activity is dependent upon the presence of two copper ions. Misfolding of tyrosinase can lead to its inability to bind copper, which results in the inactivation of the enzyme.

As a model, tyrosinase is attractive because of its long in vivo processing time in the ER (*9*), which facilitates

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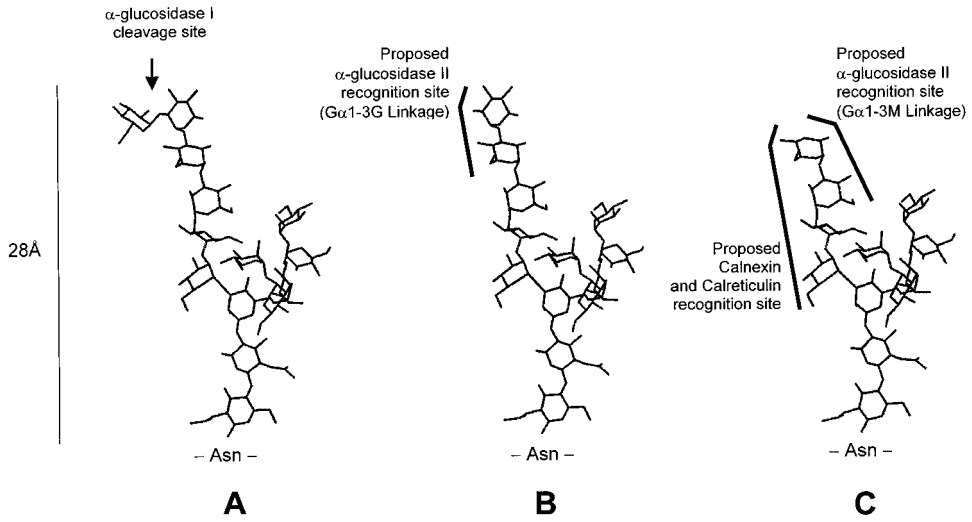

FIGURE 1: Molecular model of $Glc_3Man_9GlcNAc_2$ and proposed recognition sites of α-glucosidase I for $Glc_3Man_9GlcNAc_2$ (A), α-glucosidase II for $Glc_2Man_9GlcNAc_2$ (B), and calnexin, calreticulin, and α-glucosidase II for $Glc_1Man_9GlcNAc_2$ (C) (*12*).

investigation of transient interactions during folding. Antigen presentation by MHC molecules is also an ER-related event. Tyrosinase-derived antigenic peptides are a model to study HLA loading and presentation mechanisms. Additionally, the study of tyrosinase also allows the later events involved in metal binding into the active site to be probed. In this review we outline the results which have been obtained for tyrosinase, which provide insight into the role of calnexin/calreticulin in glycoprotein folding in vivo.

Early Stages of N-Glycan Processing Involved in Protein Folding

The nascent polypeptide chain translocated into the ER undergoes co- and posttranslational modifications including N-glycosylation, disulfide bond formation, and, in the case of multisubunit proteins, oligomerization. N-Linked glycans are attached cotranslationally to Asn residues in Asn-X-Ser/Thr motifs (where X cannot be Pro) while the polypeptides are being translocated into the ER as a core unit of 14 monosaccharide residues ($Glc_3Man_9GlcNAc_2$) (*10*).

The three glucose residues are situated at the end of the α1−3 arm of the oligosaccharide core. The overall structure of this arm is extended and the number of conformers reduced, as revealed by the statistical analysis of glycosidic linkages performed on a large database (*11*). Solution NMR studies of $Glc_3Man_7GlcNAc_2$ (*12*) revealed that the triglucosidic cap forms a tight loop that exposes the α1−2 linkage between the first two glucoses for a rapid trimming by α-glucosidase I (Figure 1A). The α-glucosidase II hydrolyzes the next two glycosidic linkages: Glcα1−3Glc and Glcα1−3Man. Configurational analysis of the NMR model indicates that these two linkages can have a common epitope starting from the carbon C6 of the first monosaccharide to the C3 of the second one. However, for the two different linkages this epitope is presented on opposite sides of the oligosaccharide (Figure 1B). By contrast, calnexin and calreticulin recognize specifically the Glcα1−3Man linkage and not the Glcα1−3Glc. As the only major structural difference between the two disaccharides consists of the epimerization at C2 of the inner residue, it follows that the Man C2 atom is involved in the interaction with the lectin. This would imply that glucosidase II and calnexin/calreticulin may approach their common substrate from opposite sides (Figure 1C). This model supports the notion that the nascent glycoprotein can form a transient ternary complex with calnexin/calreticulin and glucosidase II. Since the recognition elements of the oligosaccharides are situated at a distance of 28 Å, well removed from the polypeptide core, this makes an ideal "hook" for the ternary complex. The existence of the ternary complex has been confirmed by other studies (*13*).

Monoglucosylated glycans that bind to calnexin/calreticulin arise not only as a result of glucosidase II digestion but also by reglucosylation of completely deglucosylated oligosaccharides by the UDP-Glc:glycoprotein glucosyltransferase (GT) (*14*). UDPglucose is transported from the cytosol into the ER (*15*, *16*) where it serves as the glucose donor in the reglucosylation reaction catalyzed by GT (*17*). It has been shown that the transferase catalyzes the glucosylation of glycans attached to incompletely folded proteins (*18*, *19*). The existence of de- and reglucosylation reactions indicates that a cycle is involved in the association/dissociation of glycoproteins with chaperones, lasting as long as the glycoproteins are incompletely folded. In this cycle GT acts as the folding sensor. Final release from the calnexin/glucosidase II/glucosyltransferase cycle occurs when the polypeptide has reached a fully folded conformation (*20*). Transport from the ER to Golgi then occurs, being facilitated in many cases by mannose lectins. It has been reported that ERGIC-53 may function as a receptor mediating the ER to ER-Golgi intermediate compartment (ERGIC) transport of soluble glycoprotein cargo (*21*).

There is still a debate as to whether calnexin/calreticulin acts only as a lectin or if it also recognizes the polypeptide moiety (*3*). It has been recently reported that calreticulin functions in vitro as a molecular chaperone for both glycosylated and nonglycosylated proteins (*22*). In contrast, the in vitro experiments performed with monoglucosylated RNase B have shown that calnexin can act exclusively as a lectin, its binding being independent of the protein conformation (*13*). Calnexin and calreticulin specifically recognize the Glcα1−3Man linkage and the two other residues on the 1,3 arm of $Glc_1Man_9GlcNAc_2$ (*23*) (Figure 1).

α-Glucosidase inhibitors, such as castanospermine, deoxynojirimycin (DNJ), and *N*-butyldeoxinojirimycin (NB-DNJ),

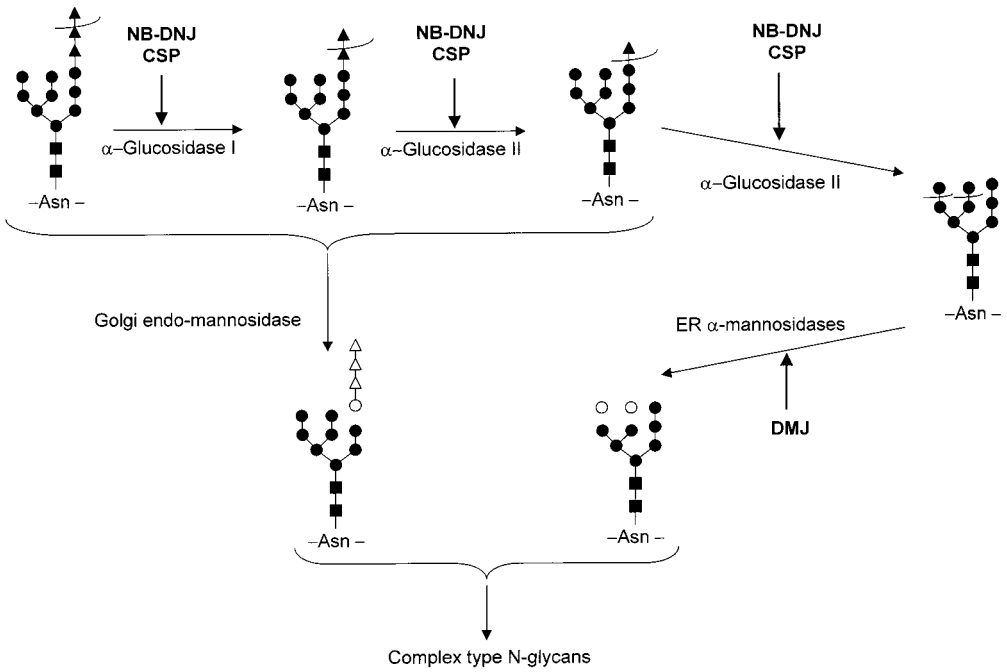

FIGURE 2: Schematic diagrams of the early stages of *N*-glycan biosynthesis. In the ER the precursor $Glc_3Man_9GlcNAc_2$ is trimmed sequentially by α-glucosidase I and α-glucosidase II. Both steps are inhibited by castanospermine (CSP) and deoxynojirimycin derivatives, such as *N*-butyldeoxinojirimycin (NB-DNJ). The Man_9 oligomannose glycoform is trimmed by ER α-mannosidases I and II and transported to the Golgi, where the glycans may be processed to complex-type sugars. These steps are inhibited by deoximannojirimycin (DMJ). The mannosidase step may be bypassed in the Golgi by an endomannosidase which removes the Glc1−3Man unit, allowing further processing of the sugar. Symbols: (●) mannose; (■) *N*-acetylglucosamine; (▲) glucose.

have been extensively used to disrupt the ER *N*-glycans processing in various systems (*24*−*26*) (Figure 2). In the presence of these α-glucosidase I inhibitors, glucose trimming is inhibited (*24*). It has been predicted that the inhibition of the calnexin interaction with glycoproteins leads to misfolded proteins that are retained in the ER and degraded (*27*). Paradoxically, many α-glucosidase inhibitors have minimal effects on cell viability and secretion (*28*), and cell lines deficient in α-glucosidases have a relatively normal phenotype (*29*). It therefore seems likely that alternative mechanisms exist within cells to enable the correct folding of many glycoproteins to occur, when they are prevented from interacting with their normal chaperones. This may be why the effects of α-glucosidase inhibition on cellular glycoproteins are selective (*30*). For example, the transferrin receptor requires correct oligosaccharide processing for cell surface expression, whereas other cell surface glycoproteins in the same cell line are expressed normally in the presence of the inhibitors (*28*). The common observation in mammalian systems is that in the presence of inhibitors some glucosylated glycoproteins fold normally (presumably those that are calnexin independent or utilize other chaperones during their folding) and some can be secreted even if they are partially misfolded (gp120 HIV), whereas others fail to fold completely and are retained in the ER prior to degradation (*31*, *32*).

Tyrosinase, the Key Enzyme of Melanogenesis

Tyrosinase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) is a melanogenic enzyme that regulates pigment synthesis in mammals (*33*). Melanogenesis is a complex metabolic pathway in which L-tyrosine is processed to the final product, melanin, by a series of oxidoreduction and isomerization reactions catalyzed by a family of enzymes known as tyrosinase-related proteins (TRPs) which are localized in the melanosomal membrane.

Tyrosinase is responsible for catalyzing the first two steps of the melanin synthesis pathway: hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and its subsequent oxidation to DOPA quinone (*34*).

Tyrosinase is a type I membrane glycoprotein with 533 amino acids, 4 occupied N-glycosylation sites, 17 cysteine residues grouped in two cysteine-rich domains, and 2 copper binding domains, copper A and copper B (*35*, *36*) (Figure 3). Some of the structural motifs found in tyrosinase including the copper B domain appear to be highly conserved, not only among tyrosinases from different species but also among the melanogenic enzymes. For example, tyrosinase and tyrosinase-related protein 1 (TRP-1) share a significant level of homology in several regions including the catalytic domain and the potential N-glycosylation sites (Figure 3). The enzymatic activity of tyrosinase is dependent upon the binding of two copper atoms in the copper A and copper B binding sites. At each of these sites three histidine residues coordinate the copper atom, and both of the copper atoms coordinate an O_2 molecule (*36*). Site-directed mutagenesis studies have revealed that three histidine residues, His 363, His 367, and His 389, are involved in the coordination of copper binding in the copper B binding domain (*37*).

Tyrosinase inborn disorders are characterized by the absence of melanin in the skin, hair, and eyes and a series of related abnormalities of the ocular system. The absence of tyrosinase activity is associated with oculocutaneous albinism (OCA) type I in many animal species, including humans (*38*). Several mutations within the tyrosinase gene

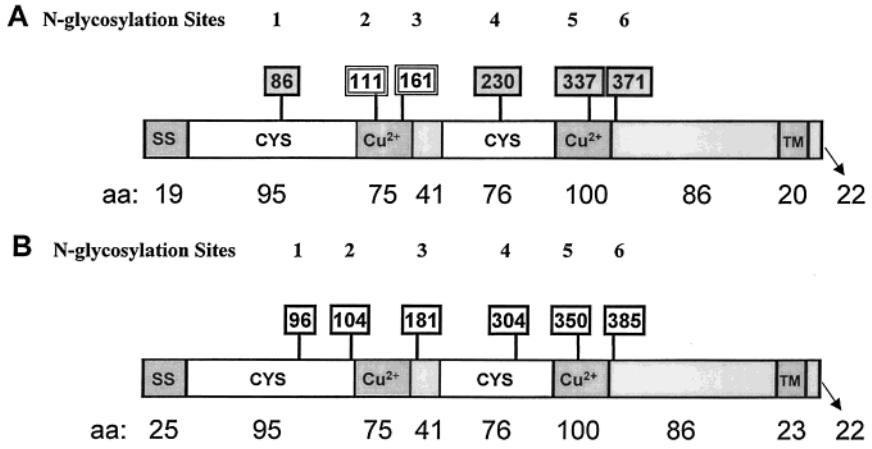

FIGURE 3: Schematic representation of tyrosinase (A) and TRP-1 (B) structures, showing the copper domains (Cu^{2+}), cysteine-rich domains (CYS), transmembrane domain (TM), and the six potential N-glycosylation sites. Sites 1, 4, 5, and 6, shown in dark shading, have been reported to be occupied in mouse tyrosinase expressed in CHO cells (*8*). Numbering is from the amino terminus of the polypetide chain including the signal sequence (SS).

of albino individuals have been reported, including a substitution in codon 371 causing a putative amino acid change from threonine (ACA) to lysine (AAA) and abolishing an N-glycosylation site (*39*, *40*). Expression of the mutant tyrosinase gene results in the synthesis of an immature protein without enzyme activity which blocks the melanin synthesis (*40*). Although the mechanism of tyrosinase inactivation in oculocutaneous albinism is not known at present, a role for this particular *N*-glycan site (site 6) (see Figure 3) in the tyrosinase folding in the presence of calnexin has been described recently and will be discussed below.

Like other membrane proteins, tyrosinase is synthesized in the ER where it acquires the correctly folded conformation, then transits to the Golgi, and is finally targeted to the melanosomes. The loss of pigmentation in metastatic melanoma cells has been reported to be accompanied by the inhibition of tyrosinase (*6*). It is believed that in amelanotic melanoma cells, tyrosinase is retained in the ER and degraded following translocation to proteasomes (*9*). In these melanoma cells, the loss of tyrosinase activity is attributed to the misfolding of the nascent chain that cannot reach destinations beyond the ER.

Tyrosinase as a Probe of the Role of Calnexin/Calreticulin

Previous studies have shown that, following treatment of B16 mouse melanoma cells with NB-DNJ (an α-glucosidase inhibitor) although correctly transported to melanosomes, tyrosinase was inactive (*6*). Tyrosinase activity can be easily measured by a DOPA oxidase assay either in a test tube or directly on a gel (Figure 4B). Analysis of mouse tyrosinase oligosaccharide sequences following treatment with NB-DNJ revealed the existence of glucosylated oligosaccharides, including $Glc_3Man_{7-9}GlcNAc_2$. Such *N*-glycan structures have also been found on gp120 expressed in the presence of NB-DNJ (*32*).

Tyrosinase does not misfold in the presence of glucosylated *N*-glycans to a degree that causes it to be retained in the ER and degraded. Instead, it is transported to its correct cellular location, namely, the melanosome. However, despite correct localization tyrosinase has virtually no catalytic activity in NB-DNJ-treated cells, and therefore the cells

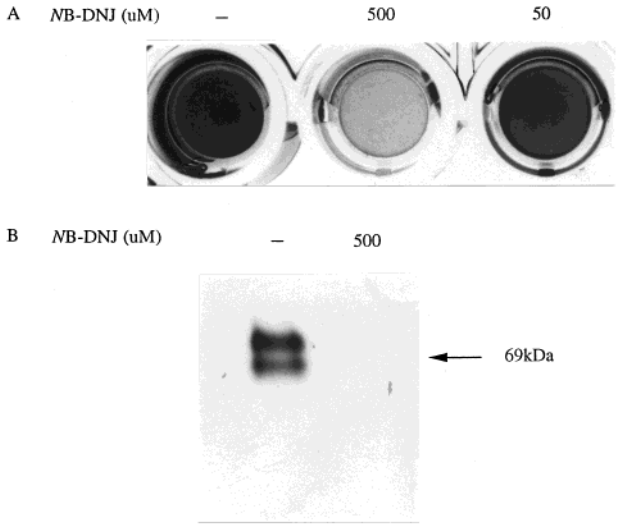

FIGURE 4: (A) Effect of NB-DNJ on B16 cells. B16 cells cultivated in the presence of NB-DNJ were transferred into a microtiter plate and pelleted by centrifugation. (B) Nonreducing SDS−PAGE of crude lysates of B16 cells cultivated in the absence (−) or presence of 0.5 mM NB-DNJ and stained for DOPA oxidase activity (*6*).

cannot support melanogenesis and are profoundly deficient in pigment relative to normal B16 cells (*6*) (Figure 4A). Similar effects have been observed on human tyrosinase. The enzyme synthesized in the human cell line MM96E, in the presence of α-glucosidase inhibitors, had no activity, and the cells were nonpigmented (*41*).

Triton X-114 extraction of the NB-DNJ-treated cells demonstrated that mouse tyrosinase is correctly incorporated in the melanosomal membrane in a fashion analogous to that of the untreated tyrosinase (*6*). For melanogenesis this is an important observation, as it has long been known that even if the enzyme is active before reaching the melanosomes, melanin synthesis is only initiated after tyrosinase insertion into the melanosomal membrane (*42*). Correct insertion of NB-DNJ tyrosinase into the membrane suggests that the depigmentation observed in treated cells is due to the inactivity of tyrosinase following from its incorrect folding rather than from its incorrect transport and localization.

Partial misfolding resulting from prevention of oligosaccharide trimming has also been observed from studies of the

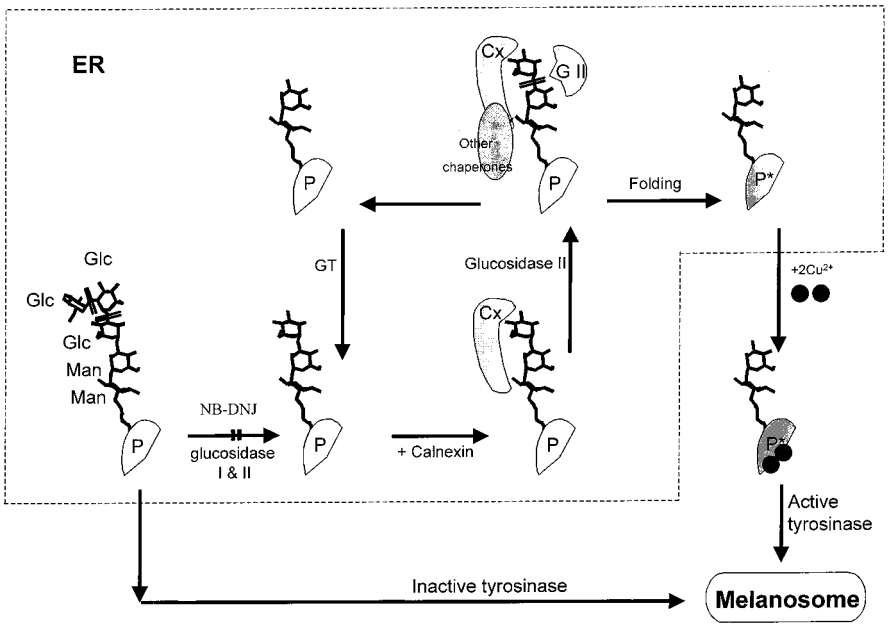

FIGURE 5: Schematic diagram of the folding and activation pathways of tyrosinase in B16 cells. $Glc_3Man_9GlcNAc_2$ glycans are added to tyrosinase polypeptide cotranslationally (represented by a single Glc_3Man_9 arm). Normally, trimming by glucosidases I and II gives $Glc_1Man_9GlcNAc_2$ glycans, allowing interaction with calnexin/calreticulin (Cx), which is part of a chaperone network. Further trimming by glucosidase II (G II) enables release of tyrosinase from calnexin and allows folding to occur. The folding state of tyrosinase is probed by UDPglucose:glycoprotein glucosyltransferase (GT). Misfolded proteins (P) are reglucosylated and recruited back into the calnexin cycle. Folded proteins (P*) continue their maturation process, which includes acquisition of two Cu^{2+}(••), and are transported to melanosomes. Pretreatment with NB-DNJ inhibits the first step and results in rapid transport of a misfolded protein (unable to acquire copper) to the melanosomes.

HIV glycoprotein gp120 expressed in NB-DNJ-treated Chinese hamster ovary cells. These showed that although regions of the gp120 are misfolded, the protein is still correctly transported to the cell surface (*43*). The structure of gp120 was probed using a panel of over 40 monoclonal antibodies. It was found that most of the regions of gp120 expressed in the presence of NB-DNJ (gp120+) were indistinguishable from gp120 expressed in the absence of the compound (gp120). However, when the conformation of the V1/V2 loops was investigated, it was found that this region was affected by the retention of glucosylated *N*-glycans. This alteration in antibody recognition was attributed to a change in the conformation of this region of the molecule. It is of interest that gp120+ transits through the ER and Golgi and is secreted from the cell at comparable rates relative to gp120 (*43*).

To determine whether the lack of complex *N*-glycans was sufficient to render tyrosinase inactive, melanoma cells have been treated with deoxynojirimycin (DMJ), an inhibitor of ER and Golgi α-1,2-mannosidases and therefore of further glycan processing (*6*) (Figure 2). There was no effect on tyrosinase activity, suggesting that the absence of complex-type *N*-glycan structures is not responsible for the maintenance of tyrosinase in an active form. Furthermore, these data suggest that retention of glucosylated high-mannose structures, due to NB-DNJ treatment, results in the loss of tyrosinase activity. To investigate whether the glucosylated glycan directly affects tyrosinase activity, the three glucose residues were removed by digestion with α-glucosidases I and II in an in vitro experiment. The digestion did not restore the enzymatic activity, showing that the glucosylated *N*-glycans do not interfere sterically with the catalytic site (*6*). The loss of activity therefore may result from partial misfolding of the protein and, in particular, of the catalytic site, showing that folding of this domain is chaperone dependent.

Recently, it has been shown that human tyrosinase activity expressed in COS cells could be enhanced by cotransfection with the cDNA coding for calnexin. When α-glucosidase activity was inhibited in the same cells by castanospermine treatment, tyrosinase activity was completely abolished (*44*). These observations indicate that calnexin/calreticulin may have a role in the correct folding of tyrosinase. This hypothesis has been independently confirmed by the detailed analysis of tyrosinase folding and interaction with calnexin in B16 cells, in both the absence and the presence of NB-DNJ (*8*). In the absence of *N*-glycan processing inhibitors tyrosinase takes ~3 h to fold correctly.

The process involves the association of at least two folding intermediates with calnexin, in the ER. Analysis of the DOPA oxidase activity, discriminating native from non-native tyrosinase forms, showed that the calnexin-bound fraction was not active (*7*). Therefore, the two observed folding intermediates are both inactive, indicating that activation of tyrosinase requiring copper loading occurs post calnexin.

Treating B16 melanoma cells with NB-DNJ determined prevention of tyrosinase binding to calnexin. As a consequence, tyrosinase folding was accelerated, and the resulting protein was more rapidly transported to melanosomes, was inactive, and did not contain copper (*7*) (Figure 5). Thus, the resulting protein should have a conformation different from that of the wild-type tyrosinase. The difference between the conformations of tyrosinase synthesized in the presence or absence of NB-DNJ must be relatively small, as the protein is not retained in the ER and is not subjected to

degradation but is correctly trafficked to the melanosomes. Most likely, rapid folding in the absence of calnexin interaction results in a conformation which is unable to accept copper.

Once the tyrosinase−calnexin complex has been formed, the inhibition of α-glucosidase II by NB-DNJ prevented spontaneous release of tyrosinase from the complex. As a result, tyrosinase folding was inhibited and tyrosinase was degraded. This suggests that α-glucosidase II activity is required to dissociate the protein from calnexin in order to allow folding to occur. This also suggests that a ternary complex between calnexin, glucosidase II, and tyrosinase should transiently occur in the living cell. Tyrosinase folding must occur off calnexin, after its release from the complex, as a consequence of α-glucosidase II activity, and before rebinding to calnexin, following reglucosylation by GT. These data also support a mechanism based on lectin-only interactions between tyrosinase and the calnexin/calreticulin cycle in living cells (Figure 5).

Thus, the role of calnexin is to promote tyrosinase-efficient folding by slowing it down, retaining the protein in the chaperone/quality control cycle, for as long as it is necessary to acquire the biologically active conformation.

Tyrosinase Glycosylation

The importance of N-glycosylation for proper functioning of tyrosinase has been clearly established by studies using N-glycosylation processing inhibitors and tyrosinase, as discussed above.

A detailed characterization of the tyrosinase *N*-glycan composition and the identification of the site occupancy have been reported recently (*8*, *45*). The expression in CHO cells of mouse tyrosinase mutants lacking single N-glycosylation sites showed that sites 1, 4, 5, and 6 at Asn residues 86, 230, 337, and 371 are fully occupied while sites 2 and 3 at Asn residues 111 and 161 are unoccupied (*8*) (Figure 3). Although the exact location has not been identified, four asparagine-linked oligosaccharide chains per molecule have also been found in hamster tyrosinase (*46*).

By sequencing the mouse tyrosinase *N*-glycans and comparing them with the data on hamster (*46*), it was found that both tyrosinases possess similar oligomannosidic series and sialylated complex antennary structures (*45*). However, the ratio of high-mannose versus complex structures is 1:3 in hamster, as compared to 1:1 in mouse tyrosinase. These data, together with the observation that mouse and human tyrosinases share 85% sequence identity and identical potential N-glycosylation sites, indicate an interesting and possibly functionally relevant conservation of N-glycosylation between tyrosinases from different species.

We should note that the members of the TRP family are all glycoproteins containing N-linked oligosaccharides. Their polypeptide chains have similar numbers of potential N-glycosylation sites (*47*), and three of them are in well-conserved positions (*48*). For instance, studies on TRP-1 and TRP-2 showed that the carbohydrate moiety of TRP-2 seems to be similar to the one of hamster tyrosinase (*49*) while TRP-1 is differently glycosylated. Both human and murine TRP-1 contain mixtures of high-mannose and complex structures. The carbohydrate analysis of TRP-1 from murine melanoma showed that only 16% of the glycans are of the high-mannose type and 16% are biantennary while 65% are processed to tri- and tetraantennary structures (*45*). This is similar to that reported for TRP-1 from human melanocytes and melanoma cells (*50*). In addition to differences between carbohydrate composition, there are substantial differences between the kinetics of maturation of tyrosinase (*51*) and TRP-1 (*52*). TRP-1 is processed in less than 1 h to its fully glycosylated form, while tyrosinase requires more than 3 h for complete maturation. Within the ER and early Golgi TRP-1 is detected for ∼30 min, whereas tyrosinase is present in the same compartments for at least 3 h (*45*). Importantly, these results have been obtained by comparing directly the kinetics of *N*-glycan processing of the two glycoproteins in the same mouse melanoma cell line. The data indicate that differences in the overall processing time of the two glycoproteins are due primarily to their ER residency and, hence, to their folding process rather than to their transport through the secretory pathway.

Tyrosinase and TRP-1 have been recently shown to behave differently in the presence of ER glucosidases inhibitors (*45*). Tyrosinase from B16 cells treated with NB-DNJ contains oligosaccharides with the $Glc_3Man_{7-9}GlcNAc_2$ structure, which indicates that no further processing of *N*-glycans occurs in the presence of this inhibitor. By contrast, TRP-1 from the same cell line is able to overcome the inhibitory effect of the ER glucosidase inhibitors and acquires oligosaccharides of complex type (*45*). Similar results were observed with TRP-1 from SK Mel-19, a human melanoma cell line treated with castanospermine or 1-deoxynojirimycin, two other inhibitors of the ER glucosidases I and II (*50*).

The glucosidase blockade can be bypassed by the use of the endomannosidase pathway, which acts in the Golgi and trims the three glucose and terminal mannose residues from the oligosaccharide moiety (*53*, *54*). Therefore, these results suggest that tyrosinase is not a substrate for endomannosidase whereas TRP-1 is. This raises the interesting and intriguing question as to whether the Golgi endomannosidase only acts on those proteins which are already correctly folded. It may well be that the misfolded proteins—as is tyrosinase in the presence of NB-DNJ—are such that the structure around the glycan moiety prohibits the access to the endomannosidase.

Individual Glycans in Tyrosinase Folding

Although it has been clearly established that N-glycosylation is essential for the correct folding of tyrosinase, a role for each individual N-linked glycan in this complex process has only recently been studied. This was done by constructing 15 tyrosinase mutants lacking one or more N-glycosylation sites using in situ mutagenesis and their transient expression in CHO cells (*8*).

The proteins were analyzed with respect to their folding rate, ability to bind calnexin, enzymatic activity, and copper content in order to characterize the folding efficiencies. The number of *N*-glycans and their location affect dramatically both the calnexin-assisted folding and the final enzymatic activity of the mutants. For instance, when three or all four occupied N-glycosylation sites are deleted, tyrosinase cannot be co-immunoprecipitated with calnexin, has no enzymatic activity, and shows an accelerated folding (*8*). Similar results have been shown for the B16 tyrosinase biosynthesized in the presence of NB-DNJ (*7*).

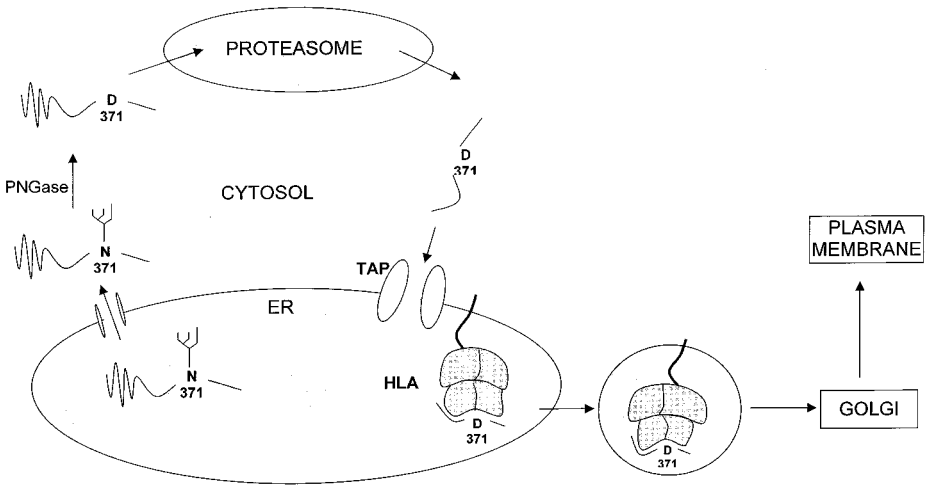

FIGURE 6: Hypothetical model for the processing and presentation of the tyrosinase antigenic peptide YMDGTMSQV (D) by class I molecules. Misfolded tyrosinase containing the YMNGTMSQV (N) peptide is exported from the ER to the cytosol where it is degraded in proteasomes following deglycosylation in the presence of PNGase, with the simultaneous conversion of Asp to Asn. The resulting peptide is transiently bound by TAP and retranslocated in the ER where it is bound by the HLA class I molecule. Upon peptide binding, the loaded class I molecule is released from the loading complex and transported through the Golgi to the cell surface.

The presence of any two occupied glycosylation sites is therefore sufficient to produce a percentage of active tyrosinase from the amount of the total synthesized protein. The amount of active tyrosinase depends on the position of the substituted sites on the polypeptide chain. For instance, the mutant Δ(4,5) (sites 4 and 5 are deleted) has wild-type activity whereas Δ(1,6) (sites 1 and 6 are deleted) is only 30% active. The presence of a specific pair of *N*-glycans located at sites 1 and 6 is crucial for correct folding of all the remaining mutants and sufficient to reproduce the wild-type activity, regardless the occupancy of the other N-glycosylation sites. This may imply that calnexin either is divalent or is acting as a dimer during its interaction with tyrosinase in vivo. Similar results were obtained when the folding of ribonuclease was analyzed in vitro. There, two glycans were needed in order to obtain stable binding of RNase to calnexin (*55*).

Removal of one or both of the two glycan sites (1 and 6) results in accelerated folding, shorter interaction time with calnexin, and lower activity of the corresponding mutants, stressing the importance of site-specific glycosylation of tyrosinase.

On the basis of these experiments, a local folding mechanism for the tyrosinase polypeptide chain has been proposed, which is regulated by the interaction with the calnexin/calreticulin cycle. In this model, individual *N*-glycans play distinctive roles depending on their location on the polypeptide chain. Regions of the protein local to some of the glycan sites may fold rapidly and correctly with limited chaperone assistance. The presence of glycans in regions of the protein structure that spontaneously fold less efficiently may be necessary for the correct function of the GT quality control system and, hence, may explain the conservation of glycosylation sites in tyrosinase. It is interesting to note that the removal of site 6 has the most significant effect on the activity of the expressed enzyme, and this is the site at which mutations have been implicated in OCA.

Copper analysis of tyrosinase mutants with partial activity suggested the presence of populations of both active and inactive tyrosinase in differing amounts. Thus, different N-glycosylation mutants show different yields of correctly folded enzyme. The population able to reach the native conformation increases with the time spent by the glycoprotein in the calnexin cycle (*8*).

It is not easy to establish a direct link between the requirements of calnexin association with tyrosinase and subsequent copper loading in tyrosinase. The components of the homeostatic mechanisms involved in copper delivery to the site of synthesis of copper-dependent enzymes are just beginning to be defined. Although some copper "chaperones" involved in copper transfer to metalloproteins have been recently identified, the details of the process remain unknown. The copper transporters identified so far belong to the P-type ATPase family, are localized in the cytosol, mitochondria, and trans Golgi network (TGN), and have been shown to deliver copper to cytochrome *c* oxidase (Cox 17), superoxide dismutase (Lys 7), multioxidase Fet3 (Atx 1), and ceruloplasmin (*56*−*58*). Another copper transporter of this family, expressed in a large number of cell lines, including B16 melanoma cells (*59*), is the Menkes protein. Patients with Menkes' disease show, besides characteristic neurological features, a peculiar appearance of the hair (pili torti) associated with hypopigmentation, implying a deficiency in the activity of tyrosinase. Interestingly, the Menkes protein has been localized in the TGN, the first compartment of the secretory pathway in melanoma cells where tyrosinase was shown to be active (*9*, *60*). Thus, these findings may further support a model in which copper transfer to tyrosinase and its activation coincide and take place in the TGN, supporting a model for tyrosinase folding occurring in two compartments.

Malignant Melanomas and Tyrosinase

The lifetime risk for developing a malignant melanoma in an American in the United States was 1 in 87 in 1996 (*61*). It is now accepted that T-lymphocytes mediate tumor-specific protective immunity (*62*). They do this by recognizing peptides which are processed intracellularly from misfolded proteins and which are then presented on the cell surface by HLA molecules. In principle, T-lymphocytes are

able to monitor and recognize any change or structural alteration occurring in somatic cells. They are particularly important in defense against viral infections. T-cell-defined antigens on human melanomas are divided into three principal categories. These are individually distinct mutated antigens, cancer antigens, and melanocyte differentiation antigens. Cancer and differentiation antigens are not altered or mutated in tumor tissues. For a variety of reasons, tumor cells are not equipped to induce primary T-cell responses since, among other factors, they also release immunosuppressive molecules. However, tumor cells can be recognized by preactivated cytotoxic T-lymphocytes which have been induced using self-peptides (differentiation antigens), either in an appropriate adjuvant or on an immunomodulatory carrier. This strategy essentially seeks to brake tolerance and is the basis for developing active therapeutic immunization strategies. Since tyrosinase is a key enzyme in melanin synthesis, its normal differentiation antigens could potentially be used in melanoma therapy.

As previously discussed, the loss of tyrosinase activity in some melanoma cells is attributed to the misfolding of the nascent chain that cannot reach destinations beyond the ER. As part of the immune defense, many melanoma epitopes are presented to cytotoxic T-lymphocytes (CTLs) by MHC class I molecules (*63*). In general, MHC class I associated peptides are derived from intracellular proteins (*64*). Interestingly, a tyrosinase peptide, YMNGTMSQV (N), corresponding to amino acids 369−377 and including the N-linked glycosylation site 6, has been shown to be presented as the converted peptide, YMDGTMSQV (D) (*65*). This peptide binds to the transporter associated with antigenic processing (TAP), which transports it into the ER. The converted peptide D probably arises as a result of the deglycosylation in the cytosol by the enzyme peptide:*N*-glycanase. This enzyme reaction also results in the conversion of Asn residues to Asp (*66*). The D peptide has also been found to be presented by HLA-A0201 on cells expressing full-length tyrosinase. Following binding of the peptide to MHC, the complex is transported through the Golgi where it is further glycosylated and then secreted to the cell surface. In general, deglycosylation of glycoproteins in the cytosol prior to degradation by the proteasome into peptides may provide a mechanism for limiting the number of peptides that can be presented. Therefore, the deglycosylation enzymes may play a crucial role in limiting the number of T-cell receptors required in the immune system.

Recent research performed using a vaccinia-encoded minigene and a cytosolic expressed tyrosinase has shown that the degradation of full-length tyrosinase occurs after translation in the ER. Moreover, presentation of the D peptide was TAP and proteasome dependent. Therefore, it has been proposed that processing of tyrosinase involves translation in the ER, export of full-length misfolded tyrosinase to the cytosol, possible deglycosylation prior to degradation, and then retranslocation of converted peptides by TAP for association with HLA (*67*) (Figure 6).

Even stronger support for this view comes from the investigation of tyrosinase synthesis in amelanotic melanoma cells. Using proteasome inhibitors, it has been shown that in these cells there is an active proteolysis of tyrosinase occurring in proteasomes (*9*). Although little is known about the factors regulating the ER-associated degradation (ERAD), it is likely that the chaperones that bind tyrosinase during its folding act in conjunction with some of the ERAD components in specific cellular conditions (*20*).

Concluding Remarks

In view of the data that we have discussed, the fundamental importance of the N-glycosylation in glycoprotein folding is demonstrated by the example of tyrosinase. Tyrosinase optimal folding is dependent on its association with the chaperones calnexin/calreticulin that recognize tyrosinase *N*-glycans and drive it into an on and off cycle in which α-glucosidase II and GT play important roles. In the case of tyrosinase, the quality control acts by selecting a conformation of the polypeptide chain able to acquire copper in its active site. Importantly, the quality control system operates locally, with individual *N*-glycans having distinctive roles.

During the past few years, it became more evident that calnexin, calreticulin, α-glucosidase II, and GT are components of a huge network of proteins, acting in a coordinated manner and recognizing specific features of the folding polypeptide. Some of these proteins may be involved in mechanisms related to the retranslocation of the misfolded glycoprotein in the cytosol and subsequent degradation. Antigenic peptides derived from tyrosinase are transported back to the ER by TAP where they interact with the class I complex MHC−HLA. Tyrosinase is an important example, therefore, which allows us to probe in detail the folding process and discover new features of the quality control system. It may also lead to a better understanding of the processing of the glycopeptides loading into MHC molecules.

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